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JUL 27 2001
PATENT & TRADEMARK OFFICE

Attorney Docket No.: 10033.200-US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Nielsen et al.

Serial No.: 09/882,144

Confirmation No: To be assigned

Group Art Unit: To be assigned

Filed: June 15, 2001

Examiner: To be assigned

For: Method for Genome Mining For Secreted Protein Genes

**CERTIFICATE OF MAILING UNDER 37 CFR 1.8(a)**

Commissioner for Patents
Washington, DC 20231

Sir:

I hereby certify that the attached correspondence comprising:

1. Claim to Convention Priority

is being deposited with the United States Postal Service as first class mail in an envelope addressed to:

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**CLAIM TO CONVENTION PRIORITY UNDER 35 U.S.C. 119**

Commissioner for Patents
Washington, DC 20231

Sir:

In the matter of the above-identified application and under the provisions of 35 U.S.C. 119 and 37 C.F.R. 1.55, Applicants claim priority of Danish application no. PA 2000 00963 filed on June 21, 2000. Applicants submit a duly certified copy of said foreign application

Respectfully submitted,

Date: July 19, 2001

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# Kongeriget Danmark

Patent application No.: PA 2000 00963

Date of filing: 21 June 2000

Applicant: Novozymes A/S
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This is to certify the correctness of the following information:

The attached photocopy is a true copy of the following document:

- The specification, claims and abstract as filed with the application on the filing date indicated above.

By assignment dated 17 Nov 2000 and filed on 01 Dec 2000, the application has been assigned to Novozymes A/S



**Patent- og**
**Varemærkestyrelsen**
Erhvervsministeriet

Taastrup 20 April 2001

Karin Schlichting
Head clerk

Modtaget
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PVS



TITLE: METHOD FOR GENOME MINING FOR SECRETED PROTEIN GENES

## FIELD OF THE INVENTION

The present invention relates to a method for mining genomes for genes encoding proteins and peptides, including enzymes, that are secreted from organisms, in particular micro-organisms such as bacteria and fungi. The method uses immunoassay techniques to identify clones expressing genes encoding enzymes or other proteins or peptides that are secreted from an organism of interest.

## BACKGROUND OF THE INVENTION

A typical bacterial genome is about 3-8 mB (mega base pairs) in size. For example, the Bacillus subtilis genome is known to have a size of 4.2 mB and to contain a total of 4100 protein coding genes. The function of 1200 gene products of Bacillus subtilis has been experimentally identified. The function of 42% of the genes could at the time the genome sequence was finished not be predicted by similarity to known genes encoding proteins with known function. These genes could be divided into three groups: 12% showed similarity to other genes with unknown function from other organisms, while 4% showed similarity to other genes with unknown function in B. subtilis only. The remaining 26% did not show homology to anything (F. Kunst et al. 1997. The complete genome sequence of the Gram-positive bacterium Bacillus subtilis. Nature 320:249-256).

Screening for enzymes, or other proteins or peptides, normally involves gene cloning in order to obtain a reasonable yield of a given gene encoding a desired product. Constructing a gene library, where the genome is cut into fragments, which are then ligated into a vector and transformed into a cloning

host, does this. If the genome of B. subtilis is randomly cut into fragments with an average size of 4 kB (kilo base pairs), at least about 1000 clones must be screened in order to cover the entire genome once. In order to ensure that all open reading frames of the genome are represented in full length, a much higher number of clones must be screened in order to ensure that the entire genome is expressed. Usually the number of screened clones is on the order of magnitude of 5000-10,000 clones.

The genomes of Aspergillus nidulans and Neurospora crassa are known to have a size of 31.0 mB and 42.9 mB of DNA, respectively (Dunn-Colemann N. & Prade, R. 1998. Toward a global filamentous fungus genome sequencing effort. Nature Biotechnology, 16, 5; Radford and Parish, 1997. The genome and genes of Neurospora crassa. Fungal Genetics and Biology, 21, 258-266). The nuclear genome of Saccharomyces cerevisiae contains 13.0 mB, and about 6200 open reading frames have been predicted (Zagulski, M., Herbert C.J. & Rytka, J 1998. Sequencing and functional analysis of the yeast genome. Acta Biochimica Polonica, 45, 627-643).

A screening for enzymes in fungi can be based on an expression-cloning method, which combines the ability of Saccharomyces cerevisiae to express heterologous (fungal) genes with the utilization of enzyme assays. The fungus of interest is fermented under conditions that give high-level enzyme activity; mRNA is prepared from the resulting biomass and a cDNA library is constructed in E. coli. Plasmid DNA is isolated from subpools of this library and transformed into S. cerevisiae. Subsequently, the yeast transformants are screened for enzyme activity.

We assume that for a fungal genome about 5000 genes are expressed. For statistical reasons, and due to the manner in

which cDNA is prepared, a high number of clones must be screened in order to ensure that all expressed enzymes are identified, i.e. on the order of magnitude of 50,000-100,000.

For a typical screen for any given enzyme or other gene product, a functional assay is applied: for example, proteases are screened in an assay specific for proteases, amylases are screened in an assay specific for amylases and so forth. The existing methods for traditional functional screening for extra-cellular enzymes are substantially limited to the applied screening assays. This means that screening of a genome provides a) only those enzymes for which a functional assay exists or can be designed, and b) only a single enzyme activity (or a very limited number of enzyme activities), i.e. the enzyme activity/activities that the assay is specific for or which can be derived from a single screening. Frequently, the same gene library is screened over and over again because it is desired to investigate several activities. This is ideally done in parallel, but as it is often not known at the outset which enzymes are of potential interest, gene libraries have to be newly constructed from the given wild type organism or the library has to be screened several times in the various functional assays. This method for screening for enzymes or other proteins has the disadvantage of being both time-consuming and expensive.

An estimate of the total number of extracellular enzymes in B. subtilis was made by 2D gel analysis of extracellular enzymes, and subsequent identification of spots by N-terminal sequencing. The number was predicted to be 150-180 extracellular enzymes (Hirose et al. 2000. Proteome analysis of Bacillus subtilis extracellular Proteins: a two dimensional protein electrophoretic study. Microbiology 146:65-75). This means that with a screening procedure designed to identify all

secreted gene products, the number of hits would be about 200 clones from the total of 4100 open reading frames. In other words, if 10,000 clones are screened, 200-500 clones will carry a DNA fragment from the original genome expressing an extra-cellular protein or peptide. With a pre-screening for clones producing extra-cellular enzymes functional screening, should, in the ideal situation, be able to be performed on these less than 500 clones.

For fungi, the number of secreted gene products is assumed to be in the range of about 500-1000 for a given genome, so that only about 500-1000 clones from a total of approximately 25,000-40,000 screened clones are of real interest.

A tremendous savings in both time and money could be achieved by mining the gene libraries or cDNA libraries for clones expressing extracellular products. In a typical example of screening of a bacterial genome, the gene library could thus be initially screened in a secretion assay in which 5000-10,000 clones are screened and the approximately 200 clones are detected that encode secreted gene products. These 200 clones could then be screened using e.g. functional assays.

This means that compared to a theoretical screening procedure based only on functional assays in which a gene library might be screened in 200 different functional assays to detect all secreted gene products (e.g. 5000 clones x 200 = 1,000,000 screened clones), in the ideal case in which clones producing secreted products may be initially identified, the gene library is screened once for secreted products (e.g. 5000 clones) and the resulting approximately 200 secreted clones can subsequently be fingerprinted for biochemical activity in functional assays. Assuming again use of the same 200 functional assays, a total of only 200 x 200 = 40,000 clones

would have to be investigated, in other words only 4% of the 1,000,000 clones that would have to be investigated using a functional assay alone.

Typically, a gene library might be screened using about 10 different functional assays. With 5000 clones this gives a total of 50,000 clones that must be screened. In the ideal case in which clones producing secreted gene products can be identified at the outset, the 5000 clones are screened once, after which all secreting clones are detected and analysed in the 10 functional assays, corresponding to a screening workload of 200 clones screened in 10 assays, i.e. only 2000 clones need to be screened in the functional assays, again a total of only 4% of the number of clones that must be screened using the functional assays alone.

For fungal cDNA libraries, the same statistical considerations apply.

In short, it would be a tremendous advantage to have a screening assay for secreted enzymes and other proteins from gene libraries wherein the relatively few clones producing secreted gene products could be identified at the outset, so that only these few clones have to be investigated in functional assays aimed at identifying proteins of interest. The present invention provides such an assay.

**SUMMARY OF THE INVENTION**

The present invention provides a novel screening method that makes it possible to screen the genome of a microorganism only once in order to identify all gene products secreted by the microorganism. Clones producing these secreted gene products may then easily and quickly be further screened for peptides or proteins having a desired function, e.g. enzymatic activity, and/or they may be subjected to nucleotide

sequencing in order to identify genes encoding compounds of interest.

In a first aspect, the invention relates to a method for screening for compounds secreted by an organism, comprising

(a) Raising antibodies against secreted products of a donor organism,

(b) providing a gene library from the donor organism,

(c) cloning the gene library into a suitable host organism,

(d) expressing the cloned genes in the host organism, and

(e) detecting positive clones, which upon expression of the cloned genes secretes a compound, using the antibodies of (a) to identify such positive clones.

The invention also in a second aspect relates to a novel compound obtained or obtainable by the method of the invention.

In a third aspect the invention relates to a method for screening for a nucleotide sequence encoding a compound secreted by an organism comprising steps (a) to (e) of the first aspect and the additional step of subjecting at least one positive clone to nucleotide sequencing to identify at least one nucleotide sequence encoding a secreted compound.

In a fourth aspect the invention relates to a nucleotide sequence obtained or obtainable by the method of the third aspect.

In a fifth aspect the invention relates to a method for screening microorganisms for strains that secrete a compound comprising step (a) of the first aspect and the additional step of subjecting the microorganism to an immunoassay using the antibodies from step (a) of the first aspect to identify microorganisms that secrete said compound.

In a sixth aspect the invention relates to a microorganism obtained or obtainable by the method of any of the fifth aspect.

**DETAILED DESCRIPTION OF THE INVENTION**

In the context of the present specification and claims, the term "secreted compound" refers to any compound that is secreted by a microorganism of interest. Secreted compounds include in particular, but are not limited to, proteins and peptides.

One object which has been solved by the present invention was to find a method for identifying secreted compounds for which no functional assay exist. Another object which has been solved by the present invention was to find a method in which several, i.e. more than one, secreted component from a donor could be identified simultaneously, thus speeding up screening methods.

As indicated above, enzymes are of particular interest. The enzymes screened for in accordance with the invention may belong to known classes of enzymes, or they may be of unknown enzyme classes, e.g. enzymes having a desired functional activity but not necessarily belonging to a known enzyme class. As used herein, the term "enzyme class" (E.C.) refers to the internationally recognized enzyme classification system, Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, Academic Press, Inc., 1992.

The types of enzymes which may appropriately be identified include oxidoreductases (EC 1.-.-.-), transferases (EC 2.-.-.-), hydrolases (EC 3.-.-.-), lyases (EC 4.-.-.-), isomerases (EC 5.-.-.-) and ligases (EC 6.-.-.-).

Preferred oxidoreductases in the context of the invention are peroxidases (EC 1.11.1), laccases (EC 1.10.3.2) and glucose oxidases (EC 1.1.3.4)], while preferred transferases are transferases in any of the following sub-classes:

a) Transferases transferring one-carbon groups (EC 2.1);

b) Transferases transferring aldehyde or ketone residues (EC 2.2); acyltransferases (EC 2.3);

c) Glycosyltransferases (EC 2.4);

d) Transferases transferring alkyl or aryl groups, other than methyl groups (EC 2.5); and

e) Transferases transferring nitrogeneous groups (EC 2.6).

A most preferred type of transferase in the context of the invention is a transglutaminase (protein-glutamine γ-glutamyltransferase; EC 2.3.2.13).

Preferred hydrolases in the context of the invention are: Carboxylic ester hydrolases (EC 3.1.1.-) such as lipases (EC 3.1.1.3); phytases (EC 3.1.3.-), e.g. 3-phytases (EC 3.1.3.8) and 6-phytases (EC 3.1.3.26); glycosidases (EC 3.2, which fall within a group denoted herein as "carbohydrases"), such as α-amylases (EC 3.2.1.1); peptidases (EC 3.4, also known as proteases); and other carbonyl hydrolases. Other preferred hydrolases are xyloglucanase, arabinase, rhamno-galactoronase, pectinases, ligninases (for example polyphenol hydrolase).

In the present context, the term "carbohydrase" is used to denote not only enzymes capable of breaking down carbohydrate chains (e.g. starches) of especially five- and six-membered ring structures (i.e. glycosidases, EC 3.2), but also enzymes capable of isomerizing carbohydrates, e.g. six-membered ring structures such as D-glucose to five-membered ring structures such as D-fructose.

Carbohydrases of relevance include the following (EC numbers in parentheses):

α-amylases (3.2.1.1), β-amylases (3.2.1.2), glucan 1,4-α-glucosidases (3.2.1.3), cellulases (3.2.1.4), endo-1,3(4)-β-glucanases (3.2.1.6), endo-1,4-β-xylanases (3.2.1.8), dextranases (3.2.1.11), chitinases (3.2.1.14), polygalacturonases (3.2.1.15), lysozymes (3.2.1.17), β-glucosidases (3.2.1.21), α-galactosidases (3.2.1.22), β-galactosidases (3.2.1.23), mannanase (3.2.1.25), amylo-1,6-glucosidases (3.2.1.33), xylan 1,4-β-xylosidases (3.2.1.37), glucan endo-1,3-β-D-glucosidases (3.2.1.39), α-dextrin endo-1,6-α-glucosidases (3.2.1.41), sucrose α-glucosidases (3.2.1.48), glucan endo-1,3-α-glucosidases (3.2.1.59), glucan 1,4-β-glucosidases (3.2.1.74), glucan endo-1,6-β-glucosidases (3.2.1.75), endo-1,4-ß-mannanase, (3.2.1.78), arabinan endo-1,5-α-L-arabinosidases (3.2.1.99), endo-1,6-ß-mannanase (3.2.1.101), lactases (3.2.1.108), chitosanases (3.2.1.132) and xylose isomerases (5.3.1.5).

However enzymes nor yet classified is particularly relevant for the present invention.

Examples of other proteins and peptides that may be screened for in accordance with the invention are: receptor binding peptides such as hormones or peptide antibiotics.

The term "organism" refers to any organism that is capable of producing and secreting compounds of interest, including micro-organisms, in particular bacteria and fungi, as well as other organisms such as cell cultures of human or other mammal or other animal or plant cells. Of particular interest in the context of the present invention are methods for screening donor strain micro-organisms for secreted compounds of interest. Once identified, the gene encoding a

secreted compound of interest can of course be transferred to any suitable host strain (typically a bacteria or fungus) for e.g. production of said compound.

The term "donor organism" refers to any of the organisms as defined above when used in the screening method of the invention to generate a gene library.

The term "host organism" refers to any of the organisms into which a gene library of a donor strain can be displayed.

The term "gene library" includes both libraries of genomic nucleotide sequences, libraries of cDNA sequences and all other types of libraries of nucleotide sequences derived from a donor organism, e.g. a genomic DNA library obtained from a donor strain in accordance with the screening method of the invention. Preferably a gene library contains the entire genomic DNA or the entire cDNA from a donor organism.

The term "primary antibody" refers to an antibody raised against a compound or a mixture of compounds secreted by the donor strain. The primary antibodies are polyclonal and recognises the compound or compounds secreted by the donor organism. In a preferred embodiment the primary antibodies are raised against compounds present in a culture supernatant and the primary antibodies recognises all the compounds in the culture supernatant. The primary antibody may be a labelled primary antibody enabling detection of presence of a compound from the donor strain directly, or the primary antibody may be recognized by secondary antibody.

The term "secondary antibody" refers to a labelled antibody specifically recognizing the primary antibodies and enables detection of bound primary antibody.

The terms "labelled primary antibody" and "labelled antibody" refer to antibodies labelled with a marker in order to detect their presence in a sample. This could be either by

emission of radiation, by an enzyme reaction or by fluorescence.

The terms "heterologous expressed compound" and "heterologous expressed DNA" refer to a protein or other compound, respectively to genes of the donor organism expressed in a heterologous host organism that could be but are nor restricted to B. subtilis, E. coli, S. cerevisia or A oryza .

The term "positive clone" refers to any host organism which (1) comprises a nucleotide sequence from a gene library derived from a donor organism under investigation, (2) upon cultivation produces and/or secretes a compound which gives an immuno-reaction with a primary antibody, thereby enabling discrimination of said positive clone from other clones that also comprises a nucleotide sequence from a gene library derived from the donor organism, but do not produce and/or secretes a compound recognizable by a primary antibody.

The term "additional screening step" refers to any screening step performed in addition to the immunoassay screening of the invention. The additional screening step may in particular be a functional assay, for example an enzyme assay, in which the function of said cloned gene is identified, or a receptor assay in which a secreted peptide binds to a receptor, or an antimicrobial assay in which an antimicrobial peptide interacts with a microorganism (bacterium or fungus).

According to the method of the invention, immunoassay techniques are used to identify clones that produce a secreted gene product. A major advantage of this method is that even unknown gene products - for example an enzyme with no known biological function - can be detected. For such an enzyme, no functional screening assay will be available, but such enzymes

will be detected in a screening directed to secreted gene products.

The invention is a method for mining genomes of genes encoding all compounds secreted by a selected donor organism.

A culture supernatant is produced by cultivation of a donor organism. Cells are separated from the supernatant. The supernatant is used for immunization, so antibodies are raised against secreted compounds produced by the donor organism. The supernatant may be used directly for this immunisation process or it may prior to the immunisation process be refined, e.g. by removing undesired compounds or adding desired compounds. In the immunisation process antibodies are raised against all compounds in the refines or unrefined supernatant. Methods for raising of antibodies, e.g. polyklonal antibodies are known to the art such as from: N. M. G. Harboe & A. Ingild:
Immunization, Isolation of Immunoglobulins and Antibody Titre Determination.
Scand. J. Immunol. Vol 17, Suppl. 10 345-351. 1983.

A gene library comprising genomic DNA, preferably the entire genome of a donor organism or cDNA of mRNA, preferably the total mRNA of a donor organism, is constructed and inserted or cloned into a suitable host organism. The recovery of nucleotide material, insertion or cloning of the gene library into the host organism may be achieved by any suitable conventional method, such as transformation or transfection of the host organism with the library. Non-limiting examples of conventional methods for recovering nucleotide materials from a donor organism include the methods described in Pitcher et al., "Rapid extraction of bacterial genomic DNA with guanidium thiocyanate", Lett. Appl. Microbiol., 8, pp 151-156, 1989; Diderichsen et al., "Cloning of aldB, which encodes alpha-acetolactate decarboxylase, an exoenzyme from Bacillus brevis",

J. Bacteriol., 172, pp 4315-4321, 1990; Dretzen et al., "A reliable method for the recovery of DNA fragments from agarose and acryla-mide gels", Anal. Biochem., 112, pp 295-298, 1981 or WO 94/19454. Non-limiting examples of conventional methods for cloning nucleotide sequences into a suitable host organism includes the methods described in Ausubel, et al. (eds.) Current protocols in Molecular Biology, John Wiley and Sons, 1995, Harwood, C. R., and Cutting, S. M. (eds.), "Molecular Biological Methods for Bacillus", John Wiley and Sons, 1990 or Sambrook et al., "Molecular cloning: A laboratory manual", Cold Spring Harbor lab., Cold Spring Harbor, NY. (1989).

Although the primary objective of the invention is to screen a host organism comprising a gene library from a donor organism for clones producing a compound which is identical to a compound produced by the donor organism itself, the screening method of the invention may also be used to screen for clones producing a compound which is not completely identical to a compound produced by the donor organism itself, but which exhibits cross reactivity with a primary antibody. Cross reactivity between an antibody raised against compound produced by the donor organism and a non identical compound of a host organism requires a certain degree of similarity, but some differences in form of mutations and/or substitutions could be allowed, while preserving the feasibility of the present invention. Accordingly the gene library from the donor organism may be subjected to manipulation before cloning it into a suitable host cell e.g. by gene shuffling, such as described by Stemmer, Proc. Natl. Acad. Sci. USA, 91, pp. 10747-10751, 1994 and Stemmer, Nature, 370, pp. 389- 391, 1994; and/or by random mutagenisis as described by Eisenstadt et al., "Gene mutation", Methods for general and molecular

bacteriology, pp. 297-316, Eds: Gerhardt P., Murray R.G.E., Wood W.A. and Krieg N.R., ASM, 1994.

Accordingly the invention encompasses screening methods wherein nucleotide sequences in the gene library of the donor has been mutated. In this context it is also possible by using the method of the invention to prepare gene libraries from other organisms than the donor organism, so as to identify secreted compounds from one or more other organisms which is sufficiently similar to secreted compounds for the donor organism to provide for cross reactivity with primary antibodies .

The clones of the host organism comprising the library are screened using the antibodies raised against the culture supernatant of the donor organism. Screening with antibodies can be performed in multiple ways, wherein, however, the basic principle is the same: 1) the antibody binds to the antigen. (2) Binding of antibody is detected. Some ways of doing this includes:

Colony hybridization:

1) clones comprising the gene library is spread on agar plates
2) clones are cultivated
3) protein or other secreted compounds are immobilised on membrane or filter
4) primary antibodies are hybridized to the filter
5) detection of bound antibody either with a labelled secondary antibody or directly with labelled primary antibody.

Methods for colony hybridisation are known e.g. from Kragelund et al.; "Outer membrane protein heterogeneity within Pseudomonas fluorescens and P. putida and use of an OprF

antibody as a probe for rRNA homology group I pseudomonads"; Applied and Environmental Microbiology, Vol. 62 (2) pp. 480-485 (1996).

Dot blot hybridization:

1) clones comprising the gene library are cultivated e.g. in micro plates.
2) supernatant or culture fluid is transferred to a hybridization membrane.
3) protein or other secreted compounds are immobilised on membrane or filter
4) primary antibodies are hybridized to the filter
5) detection of bound antibody either with a lablled secondary antibody or directly with labelled primary antibody.

Methods for dot blot ybridisation are known e.g. from Hawkes et al.; "A Dot Immuno Binding Assay for Mono Clonal and other Antibodies"; J. Analytical Biochemistry, Vol. 119 (1) pp. 142-147 (1982)

ELISA method a

1) clones comprising the gene library are cultivated e.g. in micro plates.
2) protein or other secreted compounds are immobilised on the micro plate
3) primary antibodies are hybridized to the filter
4) detection of bound antibody either with a labelled secondary antibody or directly with labelled primary antibody.

ELISA method b

1) clones comprising the gene library are cultivated e.g. in microplates.
2) primary antibodies are bound to another micro plate
3) supernatant or culture fluid with protein or other secreted compounds added to the plate coated with antibodies
4) detection of bound protein or alternative compound with labelled primary antibodies.

Methods for ELISA are known e.g. from Kirkegaard & Perry Laboratories. 1999 Product catalog. p. 66

Protein micro array:

1) clones comprising the gene library are cultivated e.g. in micro plates.
2) protein or other secreted compounds in the supernatant are immobilised on a microscope slide coated with Nylon or nitrocellulose
3) primary antibodies are hybridized to the filter
4) detection of bound antibody either with a labelled secondary antibody or directly with labelled primary antibody.

Methods for Protein micro array are known e.g. from Lueking et al.; "Protein microarrays for gene expression and antibody screening" ; Analytical Biochemistry, Vol. 270 (1) pp. 103-111 (1999)

After screening the clones comprising the gene library, positive clones may be subjected to nucleotide sequencing using methods known per se in the art. The open reading frames of the corresponding inserts are determined and the corresponding gene identified. The gene may then be identified as falling into a known class of genes for which the

functionality of the gene products are known, or it may encode an unknown secreted product. Compared to the currently applied approach of whole genome sequencing, the method of the invention provides significant advantages in terms of speed and economy.

Methods for nucleotide sequencing are known in the art e.g. from Lee et al.; "New energy transfer dyes for DNA sequencing"; Nucleic Acids Research, 1997, vol. 25, No.14.

Further after the screening, the identified positive clones may be subjected to additional screening steps e.g. to verify the screening results and/or to achieve more specific differentiation between the positive clones. For verification the positive clones may be subjected to at least one additional screening comprising cultivating said positive clones and assaying them in a second immunoassay using the same antibodies as used in the first immunoassay to eliminate possible false positives.

Additional screening steps to achieve differentiation between the positive clones includes subjecting the clones to functional assays, wherein functional properties of produced compounds are investigated. For enzyme compounds preferred functional properties includes but is not limited to wash performance, thermal stability, substrate specificity, catalytic turnover, oxidation stability, sensitivity to inhibitors, pH optimum, detergent stability, stability against microbial inactivation etc. For receptor binding peptides or peptide antibiotics preferred functional properties includes but is not limited

toxicology, distribution profile in the human or animal body, metabolisation products, side effects, rate of metabolisation or secretion, receptor binding capacity, antimicrobial capacity etc. Preferably additional screening steps are

performed using as a starting material to be tested a supernatant obtained from cultivating positives clones or a refined and/or purified product thereof.

Enzymes and other proteins with unknown functions can prove to be of significant value in order to solve important needs. For example, in relation to enzymes used in laundry detergents, certain stains are known to be removable with known enzymes. However, it is desirable to be able to identify new enzymes or proteins that could be useful for removing such stains or other stains, although for this purpose the chemical nature of the stain is not necessarily known and the biochemical nature of the enzyme does not need to be known. By using a random approach based on identifying secreted enzymes or proteins in accordance with the invention, all secreted enzymes and other proteins of a given genome can be tested directly in a stain removal assay. This assay may for practical reasons not be applicable as a direct screening assay. Since the number of enzymes to be tested in this manner is very limited compared to the total number of gene products in the genome as a whole, the functional assay used to identify enzymes with a desired activity can be performed quickly and easily.

In relation to baking or other complex food processes enzymes may on the one hand catalyze reactions leading to useful products either in terms of taste, smell or texture. On the other hand enzymes may as well remove substrates that gives unwanted properties such as bad smell or odour or structure. These reactions are not always obvious, and substrates and products are not necessarily known or may even be impossible to characterize. High throughput assays are not possible to do for such reactions. For this reason a pre-selection as disclosed herein is necessary.

In relation to animal feed enzymes may facilitate digestion and make feed better accessible or may even release useful compounds for the animal. Such assays may demand testing on animals. Also here a pre-selection is necessary in order to reduce the number of tested animals.

In relation to textile, it is well known that various hydrolytic enzymes are useful in the processing of especially cotton fabrics and yarn. The structure and the components of cotton are not fully known or understood, and for this reason enzymes of unknown function are useful. However, throughput in such tests is limited and again pre-selection is demanded.

A screening approach that detects secreted gene products in an initial step has a strong impact on the speed of the screening, the throughput needed, and the machinery and resources needed. Furthermore, this screening approach can also have a substantial impact on the nature of the encoded gene products to be identified. For example, a beneficial effect may be obtained where gene libraries from more that one genome are constructed and screened collectively, e.g. in environmental samples. It has been shown that a small sample of soil (e.g. 1 g) typically contains a variety of different microorganisms, containing both cultivable and so-called non-cultivable organisms (where the term "non-cultivable" refers to organisms that cannot be cultivated in any known medium) corresponding to 4000 different genomes (Torsvik et al. 1990. Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. Appl. Environ. Microbiol. 56:776-781). Typically, several hundred thousand clones are screened in a given functional assay to detect clones carrying a desired activity. Again, by using a secretion assay according to the invention, organisms, which is different from a donor organism from which secreted compounds antibodies are raised,

which secretes similar compounds can be identified and in case of screening of gene libraries from such organisms a significantly lower number of clones need to be screened, which can have a drastic effect on the throughput and workload.

The above-described system could also be applied to a consortium of organisms subjected to a multi-resistant pathogenic bacterium. Under such conditions, antimicrobial agents would be induced. Subsequently, the method could selectively mine the library of the entire consortium for clones expressing antimicrobial agents.

The above-mentioned system could be applied for a consortium of organisms subjected to a bacterium that might be multi resistant to known antibiotic and might be pathogenic to humans animals or plants. Under such conditions antimicrobial agents would be induced. Subsequently the method could selectively mine the library of the entire consortium for clones expressing antimicrobial agents. It is impossible to perform such tests with the extremely high throughput of a normal procedure. However, it will be possible by using the antibody mining disclosed here.

With cell cultures the method can be applied for mining for potential receptor binding compounds.

Summarizing, the present invention provides a new approach in which e.g. a bacterial or fungal genome is screened only once in order to detect all secreted gene products. These secreted gene products can then be subjected to additional assays, such as specific functional screening assays, to identify clones of interest and for e.g. identifying novel compounds with a desired activity.

The invention will be further illustrated in the following non-limiting examples.

**EXAMPLES**

Example 1: Screening for multiple proteins by colony hybridisation

Production of antibodies

A polyclonal antibody against all extra-cellular enzymes from a bacterium, Bacillus agaradhaerens strain AC13, was made by cultivation of the strain in BPX medium supplemented with 100 mM $NaHCO_3$ at 30°C for 5 days with agitation at 250 RPM (BPX medium is: potato flour 100 g/l, barley flour 50 g/l, sodium caseinate 10 g/l, soy-bean cake 20 g/l, $Na_2HPO_4$,$12H_2O$, 9 g/l and Pluronic 0.1 ml/l autoclaved in 100 ml aliquots for 40 min at 120°C). The supernatant of 50 ml of the culture was recovered by centrifugation at 3000 RPM, and concentrated by freeze drying. Subsequently, the freeze dried protein was re-suspended in 10 ml of water, sterile filtered and used for raising antibodies in rabbit (The immunisation, and recovery of antibodies provided by DAKO, Copenhagen, Denmark). This antibody is referred to as "primary antibody".

Genomic DNA Preparation

Bacillus agaradhaerens strain AC13 was propagated in LB medium to which 100 mM $NaHCO_3$ had been added. After 16 hours of incubation at 30°C and 300 RPM, the cells were harvested, and genomic DNA was isolated by the method described by Pitcher et al. (Pitcher, D. G., Saunders, N. A., Owen, R. J . 1989. Rapid extraction of bacterial genomic DNA with guanidium

thiocyanate; Lett. Appl. Microbiol. 8:151-156). (LB is: 25 g/l of LB bouillon Merck Art. 0285; for agar 15 g/l of Bacto-agar Difco Art. 0140 is added prior to autoclaving).

Genomic Library Construction

Genomic DNA was partially digested with restriction enzyme Sau3A and size-fractionated by electrophoresis on a 0.7% agarose gel. Fragments of between 2 and 7 kB in size were isolated by electrophoresis onto DEAE-cellulose (Dretzen G, Bellard M, Sassone-Corsi P, Chambon P. 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels; Anal. Biochem. 112 295-298). Isolated DNA fragments were ligated to BamHI digested pSJ1678 plasmid DNA.

Ligated DNA was used in electroporation of E. coli SJ2, or B. subtilis PL 3350. The transformed cells were plated onto LB-agar plates containing 10 ppm (for E. coli) or 6 ppm (for B. subtilis) chloramphenicol, and the agar plates were incubated for 18 hours at 37°C.

Colony hybridisation

The resulting colonies were blotted to a nitrocellulose filter (Schleicher & Schuell Art. 10401116) by incubating the agar plate with the filter on top for 2 h at room temperature. The filter was incubated for 10 min in a blocking solution, incubated in washing buffer pH 10 to which 1000x diluted primary antibody had been added, washed three times in washing buffer, incubated in washing buffer to which 1000x diluted anti-rabbit antibody (secondary antibody, Art. No. P0217, DAKO, Copenhagen) conjugated with horse radish peroxidase (HRP) had been added, washed three times in washing buffer, washed once in acetate buffer pH 5.5, and incubated in

staining solution to develop colour. All these steps were carried out at room temperature and with agitation, 50 RPM. Positive clones were detected by the formation of red spots on the filter.

Solutions for colony hybridization
Washing buffer: TRIS Merck Art. 108382, 6 g/l; Sodium chloride Merck Art. 106404, 8.75 g/l and Tween 20 Merck Art. 822184, 0.5 g/l in deionised water, pH 10

Blocking solution: Tween 20 Merck Art. 822184, 20 g/l in washing buffer

Acetate buffer pH 5.5: Sodium acetate Merck Art. 106267, 6.8 g/l, and acetic acid Merck art. 100063, 1.8 ml/l in deionised water.

Staining solution: 3-amino-9-ethylcarbazole stock 40 ml/l, Hydrogen peroxide Merck Art. 7209, 0.5 ml/l.

3-amino-9-ethylcarbazole stock: 5 g/l of 3-amino-9-ethylcarbazole Sigma Art. A-5754 in absolute ethanol.

Positive clones were recovered from the agar plate and fermented in LB broth containing 6 ppm chloramphenicol for 2 days at 37°C with agitation at 250 RPM. Cells were removed by centrifugation, 5 ml supernatant was loaded onto a 10% Bis-Tris gel (Novex Art. P0217). The gel was run as recommended by the manufacturer. The gel was then blotted to a nitrocellulose filter (BA85 Protran, Schleicher & Schuell) using a semi-dry blotter (Semi dry blotter II, Kem-En-Tec, Denmark). The filter

was hybridised and stained as described above. A positive hit was seen as a discrete band on the filter.

Example 2: Screening for multiple proteins with dot blot hybridisation of supernatants in micro-plates

The DNA library of Example 1 was plated out and cultivated as described above. Colonies were picked from plates and transferred to micro-plates using an automated colony picker (Flexys colony picking station, Genomic Solutions Inc., Ann Arbor). The DNA library was grown in micro-plates as described for liquid fermentation above.

The plates were centrifuged and supernatants were transferred to a nitrocellulose filter. The filter was hybridised and stained as described above. Samples of 1 1 of culture supernatants from cultures grown in TY broth for 1 to 3 days were loaded to the filter. Red spots on the filter indicated a reaction with the antibody.

Example 3: Screening for multiple proteins with protein arrays

The DNA library of Example 1 was plated out and cultivated as described above. Colonies were picked from the plates and transferred to micro-plates using an automated colony picker (Flexys colony picking station, Genomic Solutions Inc, Ann Arbor). The DNA library was grown in micro-plates as described for liquid fermentation above. These plates were then centrifuged at 3000 RPM and supernatants were transferred to new micro-plates. The micro-plate supernatants were spotted onto a nitrocellulose-coated microscope slide (Oncyte Art. No. 70332, Electron Microscopy Sciences, Fort Washington,

Pennsylvania) using an arrayer (GMS417, Affymetrics, Santa Clara, California). The array was hybridised as described above, except that the secondary antibody was anti-rabbit antibody labelled with Alexafluor-546 (Art. No. A-11035, Molecular Probes) The array was read using an array scanner (GMS418, Affymetrics, Santa Clara, California). The sensitivity of this method was improved by making a normalisation to the total amount of protein loaded at the array using Sypro orange (Molecular Probes Art. S-6650) staining. The array was incubated for 5 minutes in Sypro orange diluted 5000x in washing buffer (see above) followed by a brief wash in washing buffer. Protein was measured as red fluorescence with the array scanner. Normalisation and identification of positive hits were made with the computer programs Genesight, Imagene, and Clonetracker (Affymetrics, Santa Clara, California).

**CLAIMS**

1. A method for screening for compounds secreted by an organism, comprising
(a) Raising antibodies against secreted products of a donor organism,
(b) providing a gene library from the donor organism,
(c) cloning the gene library into a suitable host organism,
(d) expressing the cloned genes in the host organism, and
(e) detecting positive clones, which upon expression of the cloned genes secretes a compound, using the antibodies of (a) to identify such positive clones.

2. The method of claim 1, wherein the secreted compound is selected from the group consisting of enzymes, other proteins and peptides.

3. The method of claim 1 or 2, wherein positive clones are isolated and subjected to at least one additional screening step.

4. The method of any of claims 1-3, wherein positive clones are subjected to at least one additional screening comprising cultivating said positive clones and assaying them in a second immunoassay using the same antibodies as used in the first immunoassay to eliminate possible false positives.

5. The method of any of claims 1-3, wherein the supernatant obtained from cultivating positive clones is used as a starting material for additional screening steps.

6. The method of any of claims 1-5, wherein the secreted product is an enzyme, and wherein at least one enzyme produced by a positive clone is isolated and tested in a functional assay for desired enzymatic activity.

7. The method of any of claims 1-6, wherein the donor strain is a microorganism, in particular a bacteria or a fungus.

8. The method of claims 1-7, further comprising the step of subjecting a secreted compound from a positive clone to an assay in which a desired functionality is tested for to identify clones that produce a compound exhibiting the desired functionality.

9. The method of claim 8, wherein the desired functionality is selected from wash performance, thermal stability, substrate specificity, catalytic turnover, oxidation stability, sensitivity to inhibitors, pH optimum, detergent stability, stability against microbial inactivation, toxicology, distribution profile in the human or animal body, metabolisation products, side effects, rate of metabolisation or secretion, receptor binding capacity, antimicrobial capacity.

10. The method of claims 1-9, wherein the preparation of a gene library of step (b) is replaced by preparing a gene library from one or more microorganisms different from the donor organism.

11. The method of claims 1-9, wherein the preparation of a gene library of step (b) includes a step of mutating a nucleotide sequence of the library.

12. A novel compound obtained or obtainable by the method of any of claims 1-11.

13. The compound of claim 12 which is selected from the group consisting of proteins and peptides.

14. The compound of claim 13, wherein the protein is an enzyme.

15. A method for screening for a nucleotide sequence encoding a compound secreted by an organism comprising steps (a) to (e) of claim 1 and the additional step of subjecting at least one positive clone to nucleotide sequencing to identify at least one nucleotide sequence encoding a secreted compound.

16. A novel nucleotide sequence obtained or obtainable by the method of claim 15.

17. A method for screening microorganisms for strains that secrete a compound comprising step (a) of claim 1 and the additional step of subjecting the microorganism to an immunoassay using the antibodies from step (a) of claim 1 to identify microorganisms that secrete said compound.

18. The method of claim 17, wherein the screened microorganism is different from the donor organism of claim 1.

19. The method of claim 17, wherein the organism to be screening is present in an environmental sample comprising a mixture of different microorganisms.

20. A novel microorganism obtained or obtainable by the method of any of claims 17-19

**ABSTRACT**

The present invention relates to a method for screening for compounds secreted by an organism, comprising

(a) Raising antibodies against secreted products of a donor organism,

(b) providing a gene library from the donor organism,

(c) cloning the gene library into a suitable host organism,

(d) expressing the cloned genes in the host organism, and

(e) detecting positive clones, which upon expression of the cloned genes secretes a compound, using the antibodies of (a) to identify such positive clones.

The invention also relates to compounds, nucleotide sequences and microorganisms identifiable by said method.